Effects of dimehypo (disodium 2-methylaminotrimethylene di thiosulfonate) on growth and cocooning of the silkworm, *Bombyx mori* (Lepidoptera: Saturnidae)

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Abstract: Dimehypo (disodium 2-methylaminotrimethylene di thiosulfonate), is an insecticide used on rice and other crops in China. However, contamination of mulberry leaves with this has been implicated in a reduction of silk production. The acute and chronic toxicity of dimehypo to *Bombyx mori* L over the partial life cycle of the organism was determined based on survival, growth and cocooning of two strains of silkworm larvae. A change in the ultrastructure of the posterior silk gland cell was also observed in this study. The results showed that the growth and development of tested larvae was impeded and their life cycle was prolonged in both strains. It was also found that dimehypo was extremely harmful to the cocooning of *B mori*. Ultrastructural evidence suggests that adverse effects of dimehypo arise as a result of changes in the biosynthesis of fibroin and in the physiological activity of the posterior silk gland cell. The maximum acceptable daily dose of dimehypo based on growth and cocooning of *B mori* is less than 1.7×10^{-6} μg day^{-1} in spring-reared larvae and less than 1.7×10^{-8} μg day^{-1} in autumn-reared larvae.

Keywords: dimehypo; chronic toxicity; growth; cocooning; *Bombyx mori*

1 INTRODUCTION

Dimehypo[†] (disodium 2-methylaminotrimethylene di thiosulfonate) an organonitrogen insecticide for use on rice and other crops, has a similar structure to nereistoxin and, in China, is replacing benzene hexachloride (BHC).[1–3] Since 1977, chemical plants in China have been producing this pesticide in large quantities.

Bioassays in both the laboratory and field have clearly shown that dimehypo is an ingested poison, contact poison or fumigant to target pests as well as having ovicidal properties.[1–4] Previous toxicological research has demonstrated that dimehypo is of moderately acute toxicity to mammals.[5–9] In rats, no obvious symptoms of toxicity were observed at a dose of 10 mg kg^{-1} day^{-1}. NOED (no observable effect dose) was 50 mg kg^{-1} day^{-1}. No teratogenicity, carcinogenicity and mutagenicity has been discovered and dimehypo does not appear to harm the reproductive ability of mammals. Therefore, dimehypo is considered to have an acceptable level of safety with regard to humans, domestic animals and crops.

Nevertheless, dimehypo is highly toxic to silkworm, *Bombyx mori* L.[10–16] In the traditional areas of silk production in China, the production and usage of dimehypo has been blamed for a reduction of silk output,[13,17] and, as a result, there is a move away from silk production. A comprehensive and systematic environmental impact assessment (EIA) of the effect of dimehypo on the silkworm and mulberry ecosystem is needed. However, research has so far been restricted to studies of acute toxicity of dimehypo to the third- and the fifth-instar larvae and the impairment of cocooning of the fifth-instar larvae.[10,15,16] No chronic toxicity data are currently available for *B mori*.

An assessment of chronic toxicity is very useful in understanding the impact of a toxicant on survival, maturation, reproduction and growth over the entire life cycle of the test organism, or some critical period within its lifespan.[4,18,19] This study evaluates the impact of dimehypo on growth and cocooning of *B mori* over the partial life cycle of the organism, and includes an electron microscopic study of the ultrastructure of the posterior silk gland.

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Table 1. Effects of dimehypo on cocooning of *Bombyx mori* surviving acute feeding plus contact test (autumn)[a]

Dose of dimehypo ($\mu g\ larva^{-1}$)	*Rate of cocooning (%)*	*Mean wet weight of cocoon ($g\ cocoon^{-1}$)*	*Mean wet weight of cocoon layer ($g\ cocoon^{-1}$)*	*Percentage of mean dry weight of cocoon layer (%)*
0	100a (0)	1.7680 (±0.059)	0.4284 (±0.042)	24.23a (±3.18)
0.0612	100ab (0)	1.2439b (±0.0043)	0.2584b (±0.040)	20.77ab (±3.92)
0.108	93 (±2.6)	1.2653bc (±0.030)	0.2854bc (±0.036)	22.56abc (±3.35)
0.217	100abd (0)	1.1037 (±0.11)	0.2285bcd (±0.020)	20.70abcd (±1.38)
0.346	88 (±2.0)	1.2266bc (±0.013)	0.2456bcde (±0.022)	20.02abcde (±1.90)
0.684	100abd (0)	1.3700cf (±0.031)	0.2860bcde (±0.0058)	20.88abcdef (±0.49)
1.20	80 (±1.7)	1.4445f (±0.050)	0.3478c (±0.045)	24.08abcdef (±2.27)

[a] Means followed by the same superscript within a column are not significantly different, $P > 0.05$.

Table 2. Effects of dimehypo on cocooning of *Bombyx mori* surviving acute feeding test (autumn)[a]

Dose of dimehypo ($\mu g\ larva^{-1}$)	*Rate of cocooning (%)*	*Mean wet weight of cocoon ($g\ cocoon^{-1}$)*	*Mean wet weight of cocoon layer ($g\ cocoon^{-1}$)*	*Percentage of mean dry weight of cocoon layer (%)*
0	100a (0)	1.7680 (±0.059)	0.4284 (±0.034)	24.23a (±3.00)
0.0612	93ab (±5.3)	1.2514b (±0.14)	0.2833b (±0.028)	22.46ab (±2.36)
0.108	77c (±6.1)	1.0337bc (±0.10)	0.1980c (±0.013)	19.15abc (±1.12)
0.217	85d (±5.2)	1.1442bcd (±0.26)	0.2485bd (±0.011)	21.72abcd (±5.14)
0.346	100ab (0)	1.1282bcde (±0.14)	0.2130cde (±0.015)	18.88bcde (±1.14)
0.684	83cd (±3.5)	1.3286bdef (±0.13)	0.2740bdf (±0.0076)	20.62abcdef (±1.55)
1.20	67 (±3.5)	1.0740bcdef (±0.12)	0.2445bdef (±0.045)	22.77abcdef (±2.78)

[a] Means followed by the same superscript within a column are not significantly different, $P > 0.05$.

2 MATERIALS AND METHODS

2.1 Chemicals

A commercial 250 g kg^{-1} dimehypo WP was provided by Liyang Chemical Plant (Liyang City, Jiangsu province). Stock solutions of dimehypo for each test were prepared by dissolving the commercial formulation in distilled water.

2.2 Test organisms

Two genetic strains of *B mori* were used: $Su_3 \times Su_4$ (reared in spring) and $Su_5 \times Su_6$ (reared in autumn). The larvae were fed on mulberry (*Morus nigra* L) leaves exclusively and maintained at ambient temperature. The partial life cycle of the organism used in this study was from the very beginning of the second larval stadium to the end of cocooning.

2.3 Acute toxicity test

An acute feeding plus contact test was conducted as follows.[20] healthy and active larvae of similar size (larval length 40(±2) mm) at the end of the fourth ecdysis in autumn were randomly selected and placed in plastic basins containing mulberry leaves contaminated with dimehypo. A series of concentrations of dimehypo were painted evenly on the back of fresh and clean mulberry leaves so as to give deposits of 0.0612, 0.108, 0.217, 0.346, 0.684 and 1.20 μg per leaf. Distilled water was brushed on the leaves used for the control. Each day, after the larvae had finished ingesting the contaminated mulberry leaves, fresh and clean leaves were fed to maintain their dietary requirements. Thus, larvae were exposed to nominal doses of 0.0612, 0.108, 0.217, 0.346, 0.684 and 1.20 μg per larva dimehypo. Three replicates, each comprising 10 larvae, were used, giving a total of 30 larvae per treatment. Larvae were observed after 24 h, when dead larvae were recorded and removed. The median lethal toxicity (LD_{50}) was calculated using the method of Spearman–Karber.[4] Larvae which survived in the treatments and controls were reared until they reached full maturity, and then their cocoons were observed.

The design of a separate acute feeding test was the same as the acute feeding plus contact test except that larvae were fed with a 'sandwich' of contaminated mulberry leaves so that the test larvae could not contact dimehypo directly. The 'sandwich' consisted of two pieces of leaf material with dimehypo applied to one side only and hot agar as a binder.

2.4 Chronic toxicity test

Healthy and active larvae of similar size (larval length 3 (±0.5) mm) at the end of the first ecdysis were randomly selected and placed in plastic containers. The test larvae were exposed to nominal doses of 1.7×10^{-6}, 1.7×10^{-5}, 3.4×10^{-5}, and 1.7×10^{-4} μg day^{-1} (spring); 1.7×10^{-8}, 1.7×10^{-7}, 1.7×10^{-6}, and 1.7×10^{-5} μg day^{-1} dimehypo (autumn), administered in the same way as in the acute feeding plus contact test (Section 2.3). Distilled water again served as the control. Fifteen larvae were used in three replicates, giving a total of 45 larvae per treatment. The period of test was from the very beginning of the

second larval stadium to the end of cocooning and the following endpoints were measured:

Duration of larval stadium and ecdysis;
Mean wet weight of larva (MWL). The third- and fifth-instar larvae were sorted into large ($\geq$1.5 g) and small ($<$1.5 g) individuals and weighed. The mean wet weights of large and small individuals were recorded separately.
Rate of cocooning;
Mean wet weight of cocoon; mean wet weight of cocoon layer;
Percentage of mean dry weight of the cocoon layer.

2.5 Ultrastructure of the posterior silk gland

The posterior silk glands were sampled from the fifth-instar larvae exposed to 1.7×10^{-4} μg day^{-1} dimehypo and the corresponding controls after 144 h. They were prefixed for 24 h using a fixative containing 2.5% glutaraldehyde and 2.0% paraformaldehyde in cold phosphate buffer (0.1M, pH 7.0–7.4).[21,22] The pre-fixed specimens were cut into blocks of approximately 1 mm^3, fixed with the same fixative and stored in the cold for 30 min, followed by a rinse in cold phosphate buffer for 2 h. Rinsed specimens were put into 1% OsO_4 in the cold phosphate buffer for 3 h, and then were shifted to phosphate buffer for 15 min. Finally, the tissue blocks were dehydrated with ethanol and embedded in Epon 812. Thin sections were cut with a LKB-V Ultrotome and were doubly stained with uranyl acetate and lead citrate. The ultrastructure of the posterior silk gland was observed using a JEM-100S Electron Microscope at an accelerating voltage of 1 000 kV.

2.6 Statistical analysis

Comparisons of the effects of dimehypo on larval stadium, ecdysis and the growth of larva were analysed by a pooled *t*-test. Effects on growth and cocooning were analysed by Duncan's Multiple Range Test. In the acute feeding plus contact test and acute feeding test, the effects of dimehypo treatments on cocooning were compared by a paired *t*-test. Differences were considered significant at $P < 0.05$ and highly significant at $P < 0.01$.[23]

3 RESULTS

3.1 Acute toxicity

After 24 h exposure there was a dose-response relationship between mortality of *B mori* larvae and dimehypo dose in both acute toxicity tests. The calculated 24 h LD_{50} values and associated 95% CI were 0.318 (0.221–0.415) μg day^{-1} for the acute feeding plus contact test and 0.251 (0.175–0.327) μg day^{-1} for the acute feeding test. Furthermore, a significant reduction in the rate of cocooning of larvae which survived in the acute feeding plus contact test was detected, although not in a dose-dependent fashion (Table 1). A significant reduction in the rate of cocooning between each treatment level and the control was also measured in the acute feeding test, although the dose response was again erratic (Table 2). In both acute toxicity tests, the mean wet weight of cocoons and mean wet weight of cocoon layers of surviving larvae were obviously reduced in all treatment levels compared with the controls. However, the percentage of mean dry weight of the cocoon layer showed no difference between each treatment level and the control in both acute toxicity tests.

Table 3. Measured duration of each larval stadium and ecdysis of *Bombyx mori* in the chronic toxicity assay[a]

Rearing season	Dose of dimehypo (μg day^{-1})	Duration of larval ecdysis (h)			Duration of larval stadium (h)		
		2nd	3rd	4th	3rd instar	4th instar	5th instar
Spring	0	Simultaneous	49 (±4.0)	63 (±2.6)	104 (±2.0)	100a (±4.4)	168 (±1.7)
	1.7×10^{-6}	Simultaneous	56 (±1.7)	76 (±7.0)	128 (±6.9)	105a (±5.0)	191 (±3.6)
Autumn	0	Simultaneous	19a (±2.6)	42 (±3.6)	56 (±1.7)	80 (±2.6)	180 (±4.0)
	1.7×10^{-8}	Simultaneous	21a (±2.6)	66 (±8.7)	75 (±3.6)	102 (±4.0)	202 (±7.2)

[a] Means followed by the same superscript within a column are not significantly different, $P > 0.05$.

Table 4. Effects of dimehypo on larval weights of *Bombyx mori* (spring) in the chronic toxicity assay[a]

Dose of dimehypo (μg day^{-1})	Mean wet weight of larva (MWL) (g larva^{-1})			Mean wet weight of large individuals (MWLI) and small individuals (MWSI) in 5th instar (g larva^{-1})		Ratio of MWLI to MWSI	Percentage of small individuals in 5th instar (%)
	3rd instar	4th instar	5th instar	MWLI	MWSI		
0	0.1600 (±0.00199)	0.8511 (±0.0446)	2.9778 (±0.121)	2.98a (±0.121)	2.98 (±0.121)	1.0 (0)	0
1.7×10^{-6}	0.1273b (±0.0198)	0.4889b (±0.0399)	2.5610 (±0.0814)	3.10a (±0.148)	1.01b (±0.165)	3.1b (±0.426)	28 (±2.65)
1.7×10^{-5}	0.1222bc (±0.0070)	0.5976bc (±0.0289)	2.1806 (±0.2970)	2.46c (±0.072)	1.04bc (±0.165)	2.4bc (±0.316)	19 (±2.65)
3.4×10^{-5}	0.1022cd (±0.0119)	0.5395bcd (±0.0396)	1.7459d (±0.0929)	2.31cd (±0.267)	0.81bcd (±0.157)	2.9bcd (±0.274)	38 (±2.00)
1.7×10^{-4}	0.1163bcd (±0.0098)	0.4975bcd (±0.0350)	1.5821d (±0.0796)	2.10cd (±0.332)	0.65d (±0.184)	3.2bcd (±1.10)	36 (±5.57)

[a] Means followed by the same superscript within a column are not significantly different, $P > 0.05$.

Table 5. Effects of dimehypo on larval weights of *Bombyx mori* (autumn) in the chronic toxicity assay[a]

	Mean wet weight of larva (MWL) (g larva^{-1})				
			5th instar		
Dose of dimehypo (μg day^{-1})	*3rd instar*	*4th instar*	*Day 4*	*Day 6*	*Day 7*
0	0.1268a (±0.0024)	—	3.9286 (±0.0078)	4.1225 (±0.11)	4.1950 (±0.0079)
1.7×10^{-8}	0.1300ab (±0.0055)	0.6466b (±0.058)	2.4522b (±0.044)	3.1826b (±0.019)	3.4130b (±0.33)
1.7×10^{-7}	0.1214abc (±0.0081)	0.6667bc (±0.015)	2.5689bc (±0.063)	3.4667bc (±0.036)	3.6756bc (±0.036)
1.7×10^{-6}	0.1267abcd (±0.0038)	0.6328bcd (±0.063)	2.4689bcd (±0.12)	3.2886bcd (±0.16)	3.4318bcd (±0.25)
1.7×10^{-5}	0.1268abcd (±0.0083)	0.6491bcd (±0.047)	2.444bcd (±0.2)	3.3022bcd (±0.13)	3.4622bcd (±0.075)

	Mean wet weight of large individuals (MWLI) and small individuals (MWSI) in 5th instar (g larva^{-1})						*Percentage of small individuals (%)*		*Ratio of MWLI to MWSI in 5th instar*		
Dose of dimehypo (μg day^{-1})	*MWLI Day 4*	*MWSI Day 4*	*MWLI Day 6*	*MWSI Day 6*	*MWLI Day 7*	*MWSI Day 7*	*3rd instar*	*5th instar Day 7*	*Day 4*	*Day 6*	*Day 7*
0	—	—	5.0 (±0.17)	4.0 (±0.1)	5.0 (±0.12)	4.5 (±0.17)	0(0)	0(0)	—	1.3 (±0.06)	1.1 (±0.03)
1.7×10^{-8}	3.4b (±0.2)	0.9 (±0.2)	4.1b (±0.1)	1.0 (±0.2)	4.3b (±0.1)	1.4 (±0.2)	0(0)	10.9b (±1.10)	3.8b (±1.1)	4.1 (±0.08)	3.1 (±0.4)
1.7×10^{-7}	3.1bc (±0.17)	0.6c (±0.1)	4.2b (±0.06)	0.7c (±0.1)	4.6bc (±0.3)	0.9c (±0.2)	0(0)	11.1bc (±2.27)	5.2bc (±0.08)	6.0c (±0.8)	5.1c (±0.9)
1.7×10^{-6}	3.3bcd (±0.1)	0.6cd (±0.1)	4.4d (±0.06)	0.7cd (±0.1)	4.6cd (±0.06)	0.9cd (±0.06)	5.0 (±0.53)	8.9bc (±1.32)	5.5cd (±0.77)	6.3cd (±1.0)	5.1cd (±0.2)
1.7×10^{-5}	3.3bcd (±0.06)	0.6cd (0)	4.5d (±0.06)	0.7cd (±0.1)	4.7cd (±0.06)	0.8cd (±0.1)	3.4 (±0.53)	15.6 (±1.92)	5.5cd (±0.1)	6.4cd (±1.0)	5.9cd (±0.8)

[a] Means followed by the same superscript within a column are not significantly different, $P>0.05$.

Table 6. Effects of dimehypo on cocooning of *Bombyx mori* (spring) in the chronic toxicity assay[a]

Dose of dimehypo (μg day^{-1})	*Rate of cocooning (%)*	*Mean wet weight of cocoon (g cocoon^{-1})*	*Mean wet weight of cocoon layer (g cocoon^{-1})*	*Percentage of mean dry weight of cocoon layer (%)*
0	100 (0)	1.7680a (0.073)	0.4284a (0.029)	24.23a (0.64)
1.7×10^{-6}	68 (2.6)	1.5412b (0.058)	0.3412ab (0.089)	22.14ab (5.14)
1.7×10^{-5}	33 (6.1)	1.7667ac (0.050)	0.3583abc (0.015)	20.28abc (1.39)
3.4×10^{-5}	1.9 (3.6)	1.7571ac (0.099)	0.4000abcd (0.11)	22.76abcd (6.42)
1.7×10^{-4}	4 (1.7)	1.4000b (0.049)	0.4000abcd (0.049)	28.57abcd (6.90)

[a] Means followed by the same superscript within a column are not significantly different, $P>0.05$.

Table 7. Effects of dimehypo on cocooning of *Bombyx mori* (autumn) in the chronic toxicity assay[a]

Dose of dimehypo (μg day^{-1})	*Rate of cocooning (%)*	*Mean wet weight of cocoon (g cocoon^{-1})*	*Mean wet weight of cocoon layer (g cocoon^{-1})*	*Percentage of mean dry weight of cocoon layer (%)*
0	100a (0)	1.4804a (0.068)	0.03149a (0.0051)	21.27a (0.69)
1.7×10^{-8}	90b (3.0)	1.3888ab (0.031)	0.2900ab (0.013)	20.88ab (0.76)
1.7×10^{-7}	87bc (3.6)	1.3701bc (0.022)	0.2782bc (0.022)	20.31abc (1.76)
1.7×10^{-6}	83bc (4.0)	1.3201bcd (0.047)	0.2810bcd (0.017)	21.29abcd (0.67)
1.7×10^{-5}	93abc (5.3)	1.2493d (0.080)	0.2753bcd (0.023)	22.04abcd (3.14)

3.2 Effects of dimehypo on duration of larval stadium and ecdysis

In both spring- and autumn-reared larvae, the duration of each larval stadium and ecdysis of *B mori* was longer than that of the controls in the chronic toxicity assay (Table 3). Compared with the control, the

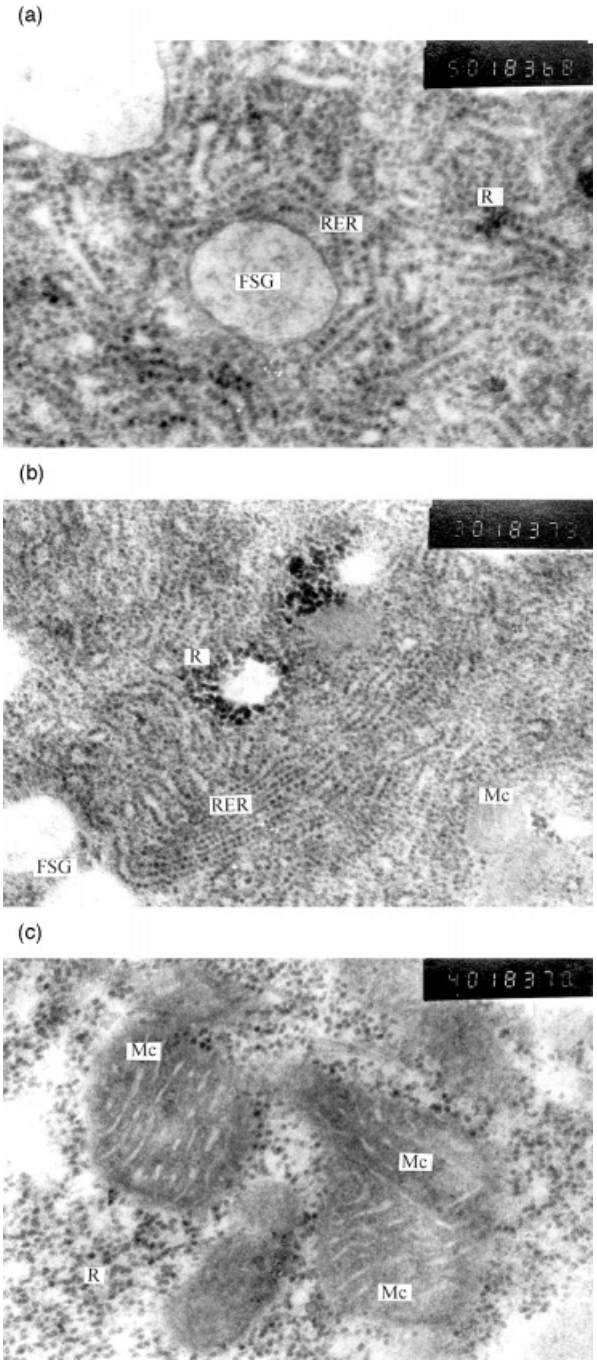

Figure 1. (a)-(c) Electron micrograph of posterior silk gland cell of the control in fifth-instar larvae at 144h. (a): × 50.000, (b): × 30.000, (c): × 40.000. RER: rough endoplasmic reticulum; FSG: secreting granules of fibroin; R: ribosome; Mc: mitochondrion.

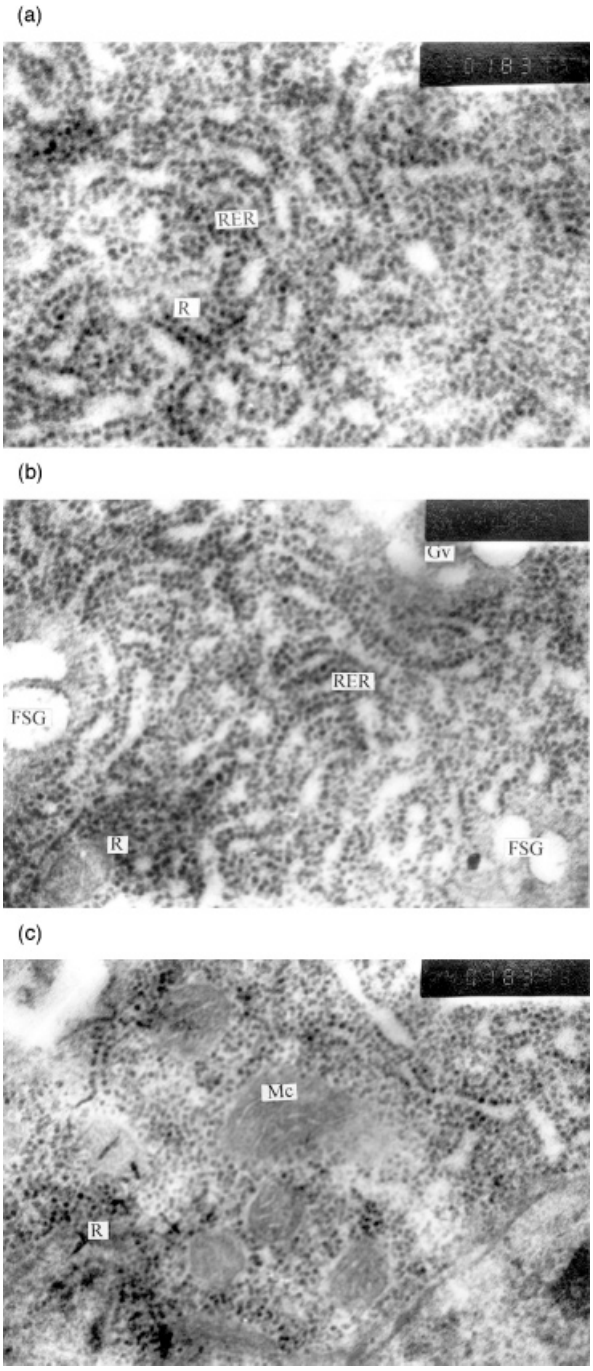

Figure 2. (a)-(c) Electron micrograph of posterior silk gland cell of the treatment group (1.7×10^{-4} μg day^{-1} dimehypo) in fifth-instar larvae at 144h. (a): × 50.000. (b): × 30.000, (c): × 40.000. RER: rough endoplasmic reticulum; FSG: secreting granules of fibroin; R: ribosome; Mc: mitochondrion.

duration of the fifth larval stadium and the fourth ecdysis were delayed by 22h and 13h (spring), 22h and 24h (autumn), respectively. These delays were highly significant (*t*-test, $P < 0.01$), except for the fourth larval stadium (spring) and the third ecdysis (autumn).

3.3 Effects of dimehypo on larval growth

Weights of *B mori* larvae in the chronic toxicity assay are shown in Table 4 (spring) and Table 5 (autumn). In spring, a significant reduction in mean wet weight of larvae (MWL) between each treatment level and controls was seen in each larval instar (*t*-test, $P < 0.05$). MWL in the third and fourth instar did not differ among treatment levels (Duncan's test, $P > 0.05$), but MWL in the fifth instar was reduced as the dose of dimehypo increased, ranging from 2.978 g larva^{-1} at 0 μg day^{-1} to 1.582 g larva^{-1} at 1.7×10^{-4} μg day^{-1} dimehypo. Furthermore, significant differences in the ratio of mean wet weight of large individuals (MWLI) to mean wet weight of small individuals

(MWSI) and the percentage of small individuals among the control and treatment levels were also observed in the fifth instar. The ratio of MWLI to MWSI and the percentage of small individuals in all treatments were higher than in the controls, but no dose-response was evident (Table 4).

In autumn-reared larvae, significant reductions in mean wet weight of larvae (MWL) in each treatment level were only measured in the fifth instar (*t*-test, $P < 0.05$) (Table 5), but MWL in the fifth instar did not obviously differ between treatment levels (Duncan's test, $P > 0.05$). Small individuals in the test population at the end of the third instar were observed only at the two highest doses (1.7×10^{-6} and 1.7×10^{-5} μg day^{-1}). However, on the seventh day in the fifth instar, small individuals were observed in all treatments. From the fourth day to the seventh day in the fifth instar, larvae exposed to a nominal dose of 1.7×10^{-8} μg day^{-1} dimehypo resulted in a significant increase in ratio of MWLI to MWSI compared with the control (*t*-test, $P < 0.05$).

3.4 Cocooning

The results concerning the effect of dimehypo on cocooning of *B mori* in the chronic toxicity assay are shown in Table 6 (spring) and Table 7 (autumn). In spring-reared larvae, the rate of cocooning was greatly reduced as the dose of dimehypo increased (*t*-test, $P < 0.01$). Whilst all larvae cocooned successfully in the controls, only 4% cocooned after exposure to 1.7×10^{-4} μg day^{-1}. Furthermore, the mean wet weight of cocoon in each treatment level was significantly less than in the controls. The lowest mean wet weight of cocoon was 1.4 g cocoon^{-1} at 1.7×10^{-4} μg day^{-1} dimehypo. Mean wet weight of the cocoon layer and percentage of mean dry weight of the cocoon layer showed no marked differences between treatments and the controls. As with the spring-reared larvae, significant reductions in the rate of cocooning between treatments and the controls were measured in autumn, but no dose-response was evident (Table 7). A nominal dose of 1.7×10^{-7} μg day^{-1} resulted in significant decreases in the mean weight of cocoons and mean weight of cocoon layers compared with the controls (*t*-test, $P < 0.05$), but, as in the spring, the percentage of mean dry weight of the cocoon layer was unaffected.

3.5 Ultrastructure of the posterior silk gland

Ultrastructures of the posterior silk gland cells in control and treatments are shown in Figs 1 and 2, respectively. In the control, cytoplasm of the gland cell was filled with well-developed rough endoplasmic reticulum (RER) of either lamellar vesicular, tubular or intermediate type (Fig 1a) and the lamellar RERs were arranged in parallel rows (Fig 1b). In addition, most of the ribosomes were attached to the membrane of ER and secretor granules of fibroin (FSG) or fibroin globules existed in large numbers in the gland cell. Furthermore, the gland cell of the control was characterised by well-developed mitochondria with circular or oval profiles and parallel cristae (Fig 1c). In contrast, the intracisternal space of RERs in the cytoplasm of the dimehypo-treated larvae was almost flattened or showed little distension. The predominant lamellar RERs were arranged chaotically and not in parallel rows. Also, fewer Golgi vacuoles were dispersed in the cytoplasm (Fig 2a, b). The size of mitochondria in the treatment group was smaller than that of the control and no parallel cristae in mitochondria was observed (Fig 2c). Obviously, subcellular structures of the posterior silk gland cell were changed in response to dimehypo treatment.

4 DISCUSSION AND CONCLUSION

Acute toxicity studies show that uptake as a result of contact is a major contributor to the acute toxicity of dimehypo, as shown by the lower LD_{50} value in the feeding plus contact test compared to the feeding test. The growth of *B mori* larvae was also greatly inhibited following chronic exposure to dimehypo: when larvae were chronically exposed to a nominal dose of 1.7×10^{-6} μg day^{-1} (spring) and 1.7×10^{-8} μg day^{-1} (autumn) dimehypo, respectively, the duration of each larval stadium and ecdysis was delayed, and mean wet weight of larvae in each larval instar was also reduced, although no significant differences were observed in the third and the fourth instar in autumn. These responses clearly showed that the development and growth of larvae treated with dimehypo were impeded and the life cycle of the tested organisms was prolonged. Furthermore, the proportion of small individuals was obviously increased at each treatment level in both spring and autumn.

Chronic exposure to dimehypo was also extremely harmful to the cocooning of *B mori*: the rate of cocooning and mean wet weight of cocoon, two indices of the spinning ability of larvae, were reduced at 1.7×10^{-6} μg day^{-1} in spring or 1.7×10^{-8} μg day^{-1} in autumn, although the mean wet weight of the cocoon layers and the percentage of mean dry weight of the cocoon layers were not affected. However, affected larvae exhibited behavioural responses, such as obstruction of cocoon-making or spinning, feeble pulse rate of the head and final death of surviving larvae. Some larvae made only thin cocoons, and loose silk was observed around larvae which failed to make cocoons. In addition, the fifth-instar larvae surviving in acute toxicity tests did not recover from the effect of dimehypo. Thus, it is clear that cocoon formation of surviving larvae was impaired by the pesticide.

Additional evidence for dimehypo effects on the cocooning of *B mori* was found by observation of the ultrastructure of the posterior silk gland cell. The posterior silk gland is a distinct tissue in *B mori* larva. The principal role of the gland is to synthesise, store and secrete fibroin which is a basic substance of silk. Tashiro *et al*[12] reported the transformation of RER from lamellar type to vesicular or tubular type

apparently proceeded in parallel with an increase in the rate of biosynthesis of fibroin and the transformation was directly correlated with the biosynthesis of fibroin. The structures of the fibroin globules (FSG) were similar to the Golgi vacuoles and their contents consisted of aggregates of native fibroin.[22] Mitochondria, the energy suppliers inside the cell, were found to be extremely vulnerable to toxic insult from extraneous substances intruding in to the cell.[24] Any toxicant interfering with normal mitochondrion operation will produce a change to physiological processes within the cell, or a clinical manifestation on the whole organism. Based on previous research and observed morphological changes of sub-cellular constituents in this study, it is suggested that the biosynthesis of fibroin and the physiological activity of the posterior silk gland cell are adversely affected by dimehypo. Biochemical changes linked to biosynthesis of fibroin and the physiological activity of the cell will be further addressed in the future, to provide biochemical evidence.

In summary, dimehypo is highly toxic to *B mori* larvae. Previous research reported a 24-h LD_{50} in an acute feeding test of 0.221 μg day^{-1} for fifth-instar larvae of *B mori* exposed to dimehypo in autumn,[10] and this has been confirmed in this study. However, the lowest observable effect dose (LOED) based on growth, development and cocooning of *B mori* in the present study was 1.7×10^{-6} μg day^{-1} in spring and 1.7×10^{-8} μg day^{-1} in autumn. The chronic toxicity of dimehypo to *B mori* was much higher than the acute toxicity. It is suggested that the maximum acceptable dose of dimehypo for *B mori* is less than 1.7×10^{-6} μg day^{-1} in spring and less than 1.7×10^{-8} μg day^{-1} in autumn. It is suggested that silk output may be reduced in regions where this pesticide is used, arising from contamination of the mulberry on which silkworm larvae feed. It is suggested that the threshold doses determined in this study should be compared with residues of dimehypo in the field following normal agricultural practice to assess the magnitude of the problem in the field and to help provide guidance on application in a way which will reduce contamination of mulberry to acceptable levels.

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